Development and validation of a gene expression test to identify hard-to-heal chronic venous leg ulcers; a three-stage cohort study

David C Bosanquet (MD)[1], Andrew J Sanders (PhD)[2], Fiona Ruge (MPhil)[2], Jane Lane (PhD)[2], Ceri A Morris (PhD)[1], Wen G Jiang (MD)[2], Keith G Harding (FRCS)[1]

[1]Clinical Innovation Hub, Cardiff University, Cardiff, CF14 4XN; [2]Cardiff China Medical Research Collaborative, Cardiff University School of Medicine, Cardiff CF14 4XN, UK

Corresponding authors:

Professor Keith G Harding; Clinical Innovation, Cardiff University, Cardiff, CF14 4XN; Email: hardingkg@cardiff.ac.uk; Telephone: +44 (0)29 2074 4505

Article type: Original article

Short Title: Genetic expression assessment of chronic wounds

Key words: chronic wounds, non-healing wounds, gene expression signature, gene transcript quantitation, stratified therapies

Role of the funding source: Funding support was provided by Welsh Government Academic Expertise For Business (A4B) Knowledge Exploitation Capacity Development LADP Scheme. No funding source was involved in study design, collection, analysis, interpretation of data, report writing, or in the decision regarding manuscript submission. The corresponding authors have full access to all data within the study and were responsible for the decision to submit the manuscript.

ABSTRACT

Background: Chronic venous leg ulcers (VLU) pose a significant burden to healthcare systems and predicting wound healing is challenging. The aim of this study was to develop a genetic test to evaluate the propensity of a chronic VLU to heal.

Methods: Sequential refinement and testing of a gene expression signature was conducted utilising three distinct cohorts of human wound tissue. The expression of candidate genes were screened using a cohort of acute and chronic wound tissue and normal skin with quantitative transcript analysis. Genes showing significant expression differences were combined and examined, using Receiver Operator Curve (ROC) analysis, in a controlled prospective study of patients with VLU. A refined gene signature was evaluated using a prospective, blinded study of consecutive patients with VLU.

Results: The initial gene signature, comprising 25 genes, could identify the outcome (healing vs. non-healing) of chronic VLUs (Area Under Curve (AUC) = 0.84, 95 per cent c.i. 0.73 to 0.94). Subsequent refinement resulted in a final 14 gene signature (WD14), which performed equally well (AUC = 0.88, 95 per cent c.i. 0.80 to 0.97). When examined in a prospective blinded study the WD14 signature could also identify wounds likely to demonstrate signs of healing (AUC = 0.73, 95 per cent c.i. 0.62 to 0.84).

Conclusion: A gene signature can identify people with chronic venous leg ulcers that are unlikely to heal.

INTRODUCTION

Successful wound healing requires the progression of a well described sequence of events; inflammation, angiogenesis, proliferation and cellular migration[1,2]. Failure of this process is not uncommon, due to a variety of systemic and local factors[3]. These resultant chronic wounds pose a significant health challenge, resulting in morbidity and a reduced quality of life[4], whilst costing the National Health Service up to 6 per cent of its annual budget[5].

Recently, an increasing volume of research has examined and documented aberrations within the cell populations surrounding chronic wounds, assuming that disturbances in normal wound healing can be traced to perturbations in gene expression following injury. Using a variety of methodologies, cellular changes have been described that are similar across a range of wound aetiologies, such as reduced cellular motility, cellular senescence, and excessive inflammation[6]. Associated alterations at a genetic level have been noted, examined either individually or collectively. Data to predict which wounds are likely to heal is lacking.

The aim of this study was to develop a wound healing score which can predict wound outcomes.[12].

METHODS

Study design

Three distinct patient cohorts were utilised to create and validate a wound-edge gene signature predictive of wound healing potential (Table 1, Figure 1). Wound edge tissues from healing/acute wounds were compared with non-healing/chronic wounds and normal skin, and molecular markers found to be significantly different were analysed further. Research ethics approval was obtained for each patient cohort, and patients were enrolled after providing informed written consent.

The 'Screening cohort'), comprised patients with acute surgical wounds following excision of pilonidal sinus disease), chronic venous leg ulcers (VLUs) and skin samples from healthy volunteers (Table S1)[13]. Some 121 potential candidate genes (Table S2) were selected for screening based on their role in cellular processes essential for successful wound healing. Genes demonstrating significant expression differences between tissue types were identified and classified as either wound healing promoters or inhibitors, whilst genes showing no difference in expression were not analysed further. The presence of a gene promoting wound healing (or the absence of a gene inhibiting wound healing; Table S3) was considered a score of one. The summation resulted in the initial gene signature score. A cut-off value was selected, with scores greater than this considered indicative of a 'healing' score.

The second retrospective cohort (the 'validation cohort') comprised patients with VLUs undergoing wound edge biopsies as part of a controlled prospective study evaluating a range of biomarkers on ulcer prognosis, as previously reported[14]. After 12 weeks of

standard best medical care, as prescribed by a senior wound healing clinician, wounds were classified as healed or non-healed. The initial gene signature score was calculated for each patient, and compared to the actual clinical outcome. Refinement of the gene signature was undertaken by assessing the prognostic ability of each individual gene. Individual genes least able to distinguish healed and non-healed wounds underwent stepwise removal, starting with the least different gene. The remaining genes, able to distinguish healed from non-healed wounds, were combined as the final gene signature (Table S4).

The third cohort (the 'study cohort'), comprised a prospective, blinded study enrolling consecutive patients with VLUs referred to a tertiary wound healing unit (Table S5)[15]. Wound edge biopsies were obtained at the initial visit. After 12 weeks of standard best medical care, as prescribed by a senior wound healing clinician, wound outcomes were recorded. Tissue specimens underwent blinded batch testing using the refined gene signature. Predicted outcomes from the gene signature were compared to actual clinical outcomes. Important clinical parameters (age, smoking status, wound duration at entry to study and wound infection during study period) known to affect wound healing were captured and examined alongside the gene signature.

Exclusion and inclusion criteria and clinical assessment of wound healing

For all cohorts, VLUs, showing clinical features consistent with underlying venous disease, were diagnosed by a senior wound healing physician at a tertiary wound healing centre. Inclusion criteria included the presence of a lower limb VLU for a minimum of three months despite standard medical care, age >18 years, and a wound size of >2cm^2 and <100cm^2.

Patients with obvious signs of infection, concomitant peripheral arterial disease (Ankle Brachial Pressure Index < 0.8), malignancy, autoimmune wounds, patients receiving systemic immunosuppression or chemotherapy and where ulcer aetiology was uncertain, were excluded. Patients in the validation and study cohort who failed to attend their follow-up clinics were also excluded. All patients in the study cohort had concurrent histological examination of their wound biopsy to exclude occult neoplastic or autoimmune disease. Wound care was personalised for each patient, with wounds being systematically treated *as per* the TIME (Tissue, Infection/Inflammation, Moisture, Edge) wound bed preparation approach[2,16]. Topical antimicrobials or antibiotics were prescribed as required in the event of an episode of clinical wound infection. Graduated multi component bandaging systems were used to compress the limb consistent with international guidelines. Wound area was assessed by specialist wound care nurses at each visit. Surgery for superficial venous incompetence was not performed in the presence of an active ulcer; patients suitable for treatment were referred to the appropriate specialist once healing was obtained.

Methods of biopsy and tissue processing

Prior to biopsy, all patients were assessed for bleeding risk and, where required, anticoagulation was stopped before the procedure. Antiseptic cleansing was used at the biopsy site and a 6mm core biopsy was taken from the wound edge, capturing both wound base and the leading keratinocyte edge, under local anaesthesia (1 per cent lignocaine). Haemostatic dressings were applied until the next dressing change. Antibiotics were not routinely prescribed.

Biopsies were immediately frozen in dry ice before being transferred to a -80^{o}C freezer until batch analysis was undertaken. Specific details of the analysis are provided in supplementary material.

Statistical analysis

Data analyses were undertaken using Minitab version 14.0 (Minitab Inc., Coventry, UK), SPSS version 18 (SPSS Inc, Chicago, Ill, USA) and SigmaPlot 11 (Systat Software Inc., London, UK) with *P* values ≤0.05 considered statistically significant. The gene transcript expression from the screening cohort were analysed using a Kruskal Wallis test. Receiver Operator Curve (ROC) area under curve (AUC) analysis of the gene signature was undertaken, and are presented with their 95 per cent confidence intervals (c.i.). Sensitivities, specificities and likelihood ratios are reported for the pre-determined signature cut off values and are given with their 95 per cent c.i.

For the study cohort, a binary logistic regression calculation was used to assess if the final gene signature and other clinical parameters, were predictive of the final clinical state. Univariate factors with a *P* value ≤0.1 were carried forward to a multivariate model, with *P*≤0.05 considered statistically significant in the final model.

RESULTS

Gene expression screening and creation of the WounD25 (WD25) gene signature

The screening cohort comprised 34 patients with acute surgical wounds following excision of pilonidal sinus disease (n=10), chronic VLUs, (n=14) and skin samples from healthy volunteers (n = 10). Interrogation of the expression of the 121 candidate genes (Table S2) identified 25 genes able to differentiate between wound types (Table S3). Based on the gene expression, each gene was defined as a promoter or inhibitor of healing and scored as either 1 or 0, as detailed above. These 25 gene scores were combined to form the WD25 (WounD25) gene signature, with a value of >16 considered predictive of healing. The WD25 gene signature was able to clearly distinguish acute from chronic wounds (ROC AUC: 0.95; 95 per cent c.i. 0.86 to 1.03), with a sensitivity of 92.9 (95 per cent c.i. 66.1 to 99.8) per cent, a specificity of 90.0 (95 per cent c.i. 55.5 to 99.8) per cent a positive likelihood ratio (PLR) of 9.29 (95 per cent c.i. 1.44 to 59.95) and a negative likelihood ratio (NLR) of 0.08 (95 per cent c.i. 0.01 to 0.53; Figure 2).

WD14 gene signature creation

The validation cohort comprised 71 participants with chronic VLUs undergoing wound edge biopsy. After 12 weeks of standard best medical care 20 ulcers had healed (completely epithelialised) whilst 51 remained unhealed. The outcomes predicted by the WD25 were compared to clinical outcome. The WD25 resulted in an AUC of 0.84 (95 per cent c.i. 0.73 to 0.94; Figure 3a/c), a sensitivity of 92.2 (95 per cent c.i. 81.1 to 97.8) per cent, a specificity of

55.0 (95 per cent c.i. 31.5 to 76.9) per cent, a PLR of 2.05 (95 per cent c.i. 1.25 to 3.35) and an NLR of 0.14 (95 per cent c.i. 0.05 to 0.40).

The WD25 signature was further refined by assessing the contribution of individual genes to the model. This resulted in the stepwise removal of a further 11 redundant genes which failed to distinguish between wound type when assessed individually. The remaining 14 genes were combined as the WD14 (WounD14) gene signature (cut off score of >8; Table S4), and the predicted outcomes of each patient were re-calculated. The WD14 gene signature was also able to distinguish between healing and non-healing chronic wounds with an AUC of 0.88 (95 per cent c.i. 0.80 to 0.97; Figure 3b/c), a sensitivity of 86.3 (95 per cent c.i. 73.7 to 94.3) per cent, a specificity of 70.0 (95 per cent c.i. 45.7 to 88.1) per cent, a PLR of 2.88 (95 per cent c.i. 1.46 to 5.67) and an NLR of 0.20 (95 per cent c.i. 0.09 to 0.41).

Predictive value of WD14 in patients with chronic venous leg ulcers as assessed in a prospective study

A prospective, blinded, open study of the WD14 gene signature was undertaken in 85 consecutive patients referred to a tertiary wound healing unit. After 12 weeks, 41 wounds were classified as 'healing' (any reduction in wound size), whilst 44 were static or enlarging and classified as 'non-healing'. Predicted outcomes from the WD14 were compared to actual outcomes. WD14 remained a significant predictive tool for assessing healing potential (AUC = 0.73; 95 per cent c.i. 0.62 to 0.84; Figure 4), with a sensitivity of 63.6 (95 per cent c.i. 47.8 to 77.6) per cent, a specificity of 85.4 (95 per cent c.i. 70.8 to 94.4) per

cent, a PLR of 4.35 (95 per cent c.i. 2.01 to 9.41) and an NLR of 0.43 (95 per cent c.i. 0.28 to 0.64).

Binary logistic univariable regression identified only the WD14 gene signature and wound duration as independent variables which predicted outcomes at 12 week follow up (Table 2). On multivariable analysis, both variables remained significant (WD14: $P \leq 0.001$ (odds ratio (OR): 12.17); wound duration: $P=0.044$ (OR: 0.98; R^2 0.38; Table 2).

DISCUSSION

This study has demonstrated the potential of a test quantifying the gene expression of a number of key molecules to differentiate between healing and non-healing VLUs. A panel of 25 genes distinguished between both acute *versus* chronic wounds and healing *versus* non-healing chronic wounds. This panel was reduced to 14 genes, without compromising diagnostic power. The WD14 gene signature was able to correctly predict clinical outcomes after three months with a sensitivity of 63.6 per cent and a specificity of 85.4 per cent.

Chronic wounds are costly and a considerable source of morbidity and reduced quality of life[4]. Grading the healing potential of a wound generally relies upon a few well recognised but relatively simplistic parameters, such as ulcer size and duration[20,21]. Sensitive prognostic markers are noticeable by their absence[22]. Gene expression testing for predicting clinical outcome and tailoring treatment has been successfully utilised for a number of cancers[11,23,24]. However, unlike cancer, wound healing remains a practice with few accurate predictive tools.

Wound edge biopsies are often utilised as part of the standard care of patients with chronic wounds, undertaken to exclude occult neoplasm or autoimmune-mediated pathology. They cause minimal morbidity, heal rapidly, and do not extend overall ulcer healing times[25]. When comparing chronic wounds with normal skin, molecular markers show much greater variability between wound edge tissue compared to wound base tissue, making these tissues preferential for genetic typing of the healing potential of wounds[9]. Given the increasing recognition that many chronic wounds are harbouring occult neoplasms[26], histological interrogation of the wound edge is likely to become commonplace in future years.

Wound healing and cancer share a number of cellular processes, including inflammation, cell growth, angiogenesis, formation of fibrous tissue/ECM, and cellular migration[17,18]. It is the control and termination of these processes that separates these two diametrically opposed processes. Previous experience in expression profiling of cancer related genes in breast and other cancers was drawn upon in the original selection of the 121 candidate genes to test as diagnostic markers of wound healing. Of these 121 genes, 25 and subsequently 14, were taken forward as a result of their differential expression within a cohort of acute and chronic wounds and normal skin to test as a combined signature for diagnostic potential.

A greater wound duration was shown to be associated with a reduced likelihood of healing at subsequent follow up and is well documented to be associated with a reduced tendency to heal across a variety of observational and randomised studies[20]. What is not clear is the causality wound duration plays, or if by definition, it is simply a marker of wounds which show a poor tendency to heal. Despite these difficulties in disentangling cause and effect,

these data on wound duration from this study provide further impetus to try and avoid chronic wounds which by definition are harder to heal. This supports an aggressive approach to wounds whilst they are still young, to try and reduce the proportion of them which develop chronicity.

Prognostic information provided by the genes identified in this study has potential clinical utility. Current outcomes for patients with chronic wounds are highly variable, and it is difficult to accurately counsel patients as to their individual prognosis. Numerous technical advances are now available to treat those with hard-to-heal wounds, including dermal substitutes, allogenic cultured skin equivalents and hyperbaric oxygen therapy, although these novel devices are generally expensive and often only accessible in tertiary centres[35]. Those with low healing potential should be targeted with more aggressive intervention in order to promote wound closure, whilst those predicted as being highly likely to heal can be managed with standard regimens. Regular and repeated debridement can also be used in those wounds with a low healing propensity, in an attempt to transform static wound edge tissues to an active healing phenotype[36]. Fully powered Randomised Controlled Trials are required to show what treatment options are of value for wounds of low healing potential as, to date, the data to demonstrate benefits of many interventions does not exist and this could be due in part to heterogeneity of the population of patients studied.

This study has some limitations. The cohort comprises patients reviewed in a tertiary wound healing unit, and its applicability in an unselected cohort of patients with wounds is unknown. Patients from the study cohort had a median wound duration of 21 months, approximately 50 per cent have deep venous disease, and were generally considered ‘hard to heal’ prior to review. The WD14 tool is therefore developed in a distinct subset of

patients with VLUs which limits translation of its usage to uncomplicated VLUs, especially when healing rates of >85 per cent over a 24 week period are reported in VLUs of <6 months duration with superficial venous disease only[37]. The WD14 would have to demonstrate much greater prognostic power in this cohort and external validation in larger cohorts of patients before it could be used in standard clinical practice. Further research is required to ascertain the most appropriate way of utilising gene signatures in the management of patients with chronic wounds. This cohort comprised VLUs alone, although chronic wounds of differing aetiologies share many similarities at the molecular and biochemical level, and it may be that the WD14 score is able to correctly identify the likelihood of wound healing for wounds of different aetiologies. Such work is under investigation, whilst also recognising the importance of validating WD14 in a larger cohort of VLUs from other centres. However, the results presented here are promising and have been validated in a sequential manner with 180 patients in total. The inefficiency in the systems of routine care provision make a strong case to support the use of tests to improve targeting of specific therapies for hard to heal ulcers, and continuation of simple remedies for wounds that have a biological propensity to heal. The current study adds to this vital area of research, highlighting WD14 as a valuable tool in helping to predict outcomes in chronic wounds, with the potential to significantly alter how treatment is prioritised in these patients, and highlighting the applicability of precision based medicine in wound healing.

TABLES

Table 1. Summary of the three cohorts used for WD14 creation and evaluation. † Of 17 samples originally collected, three samples had insufficient RNA/cDNA available to be analysed.

	Cohort design	Number of patients	Genes examined	Outcome
Screening Cohort (Ethical Approval Reference Number 04/WSE02/10)	Retrospective analysis of tissue bank	34: acute wounds = 10; VLUs = 14†; normal skin = 10	121 candidate genes (Table S2)	25 genes expressed differently between wound type - combined as initial WD25 gene signature
Validation cohort (Ethical Approval Reference Number SJT/C617/08)	Retrospective analysis of tissue bank	71 VLUs; 20 healed at 3 months, 51 non-healed at 3 months	WD25 (Table S3)	11 genes which failed to differentiate between healed and unhealed wounds were removed. Resultant 14 genes combined as WD14 gene signature
Study cohort (Ethical Approval Reference Number 09/WSE02/51)	Prospective controlled trial	85 VLUs; 41 healing at 3 months, 44 static or non-healing at 3 months	WD14 (Table S4)	Prognostic power of WD14 gene signature determined

Table 2. Univariate and multivariate analysis of wound duration and WD14 gene signature score. The WD14 gene signature was significantly predictive of healing outcomes on multivariate analysis. A greater wound duration was associated with a reduced likelihood of subsequent healing. NA: Not applicable.

Factor	**Univariate**		**Multivariate**	
	***P* value**	**Odds ratio (95 per cent c.i.)**	***P* value**	**Odds ratio (95 per cent c.i.)**
Wound duration	0.053	0.985 (0.970-1.000)	0.044	0.983 (0.966-0.999)
WD14 gene signature score	<0.001	9.529 (3.315-27.396	<0.001	12.166 (3.800-38.949)
Age	0.284	0.985 (0.959-1.012	NA	
Smoking	0.119	0.317 (0.075-1.344)	NA	
Use of antibiotics or antimicrobials	0.146	2.153 (0.766 – 6.054)	NA	

ACKNOWLEDGEMENTS

The authors wish to thank the Welsh Government Academic Expertise For Business (A4B) Knowledge Exploitation Capacity Development LADP Scheme and the help given by Dr Eryl Francis of Cardiff University's Research and Innovation Services Department. The authors are also grateful to Dr Kim Cox for their contribution to the statistical analysis.

Because of the sensitive nature of the data collected for this study, requests to access the dataset from qualified researchers trained in human subject confidentiality protocols may be sent to the corresponding author.

No preregistration exists for the reported studies reported in this article.

AUTHORS CONTRIBUTIONS

W.G.J and K.G.H designed and oversaw the study. D.C.B, K.G.H, and C.M were involved in collections and documentation of clinical materials used in the study. D.C.B, A.J.S, F.R, J.L, and W.G.J were involved in the processing of materials and in conducting experiments. W.G.J, K.G.H and D.C.B were involved in the analysis of data. W.G.J and K.G.H were involved in study concept and design. D.C.B, A.J.S, W.G.J, K.G.H wrote the manuscript. All authors were involved in proofing and correction of the manuscript.

DECLARATION OF INTERESTS

D.C.B, A.J.S, F.R, J.L, C.M, W.G.J and K.G.H have no interests to declare. The authors wish to highlight that a patent relating to the reported genetic test has been filed by University College Cardiff Consultants Limited, wholly owned by Cardiff University.

REFERENCES

1. Antsiferova M, Werner S. The bright and the dark sides of activin in wound healing and cancer. *J Cell Sci* 2012; **125**: 3929-37.
2. Harries RL, Bosanquet DC, Harding KG. Wound bed preparation: TIME for an update. *Int Wound J* 2016; **13**: 8-14.
3. Harding KG, Morris HL, Patel GK. Science, medicine, and the future - Healing chronic wounds. *Br Med J* 2002; **324**: 160-63.
4. Price PE, Fagervik-Morton H, Mudge EJ, Beele H, Ruiz JC, Nystrom TH*, et al.* Dressing-related pain in patients with chronic wounds: an international patient perspective. *Int Wound J* 2008; **5**: 159-71.
5. Phillips CJ, Humphreys I, Fletcher J, Harding K, Chamberlain G, Macey S. Estimating the costs associated with the management of patients with chronic wounds using linked routine data. *Int Wound J* 2016; **13**: 1193-97.
6. Medina A, Scott PG, Ghahary A, Tredget EE. Pathophysiology of chronic nonhealing wounds. *J Burn Care Rehabil* 2005; **26**: 306-19.
7. Stojadinovic O, Pastar I, Vukelic S, Mahoney MG, Brennan D, Krzyzanowska A*, et al.* Deregulation of keratinocyte differentiation and activation: a hallmark of venous ulcers. *J Cell Mol Med* 2008; **12**: 2675-90.
8. Cole J, Tsou R, Wallace K, Gibran N, Isik F. Early gene expression profile of human skin to injury using high-density cDNA microarrays. *Wound Repair Regen* 2001; **9**: 360-70.
9. Kirsner RS, Charles CA, Tomic-Canic M, Vincek V, Nassiri M, Stojadinovic O*, et al.* A gene signature of nonhealing venous ulcers: Potential diagnostic markers. *J Am Acad Dermatol* 2008; **59**: 758-71.
10. Mannello F, Ligi D, Canale M, Raffetto JD. Omics profiles in chronic venous ulcer wound fluid: innovative applications for translational medicine. *Expert Rev Mol Diagn* 2014; **14**: 737-62.
11. Paik S, Shak S, Tang G, Kim C, Baker J, Cronin M*, et al.* A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. *N Engl J Med* 2004; **351**: 2817-26.
12. Hamburg MA, Collins FS. The path to personalized medicine. *N Engl J Med* 2010; **363**: 301-4.

13. Conway K, Ruge F, Price P, Harding KG, Jiang WG. Hepatocyte growth factor regulation: an integral part of why wounds become chronic. *Wound Repair Regen* 2007; **15**: 683-92.

14. Moore K, Hall V, Paull A, Morris T, Brown S, McCulloch D*, et al.* Surface bacteriology of venous leg ulcers and healing outcome. *J Clin Pathol* 2010; **63**: 830-4.

15. Bosanquet DC, Harding KG, Ruge F, Sanders AJ, Jiang WG. Expression of IL-24 and IL-24 receptors in human wound tissues and the biological implications of IL-24 on keratinocytes. *Wound Repair Regen* 2012; **20**: 896-903.

16. Schultz GS, Barillo DJ, Mozingo DW, Chin GA. Wound bed preparation and a brief history of TIME. *Int Wound J* 2004; **1**: 19-32.

17. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med* 1986; **315**: 1650-9.

18. Schafer M, Werner S. Cancer as an overhealing wound: an old hypothesis revisited. *Nat Rev Mol Cell Bio* 2008; **9**: 628-38.

19. Bosanquet DC, Ye L, Harding KG, Jiang WG. Expressed in high metastatic cells (Ehm2) is a positive regulator of keratinocyte adhesion and motility: The implication for wound healing. *J Dermatol Sci* 2013; **71**: 115-21.

20. Bosanquet DC, Harding KG. Wound duration and healing rates: cause or effect? *Wound Repair Regen* 2014; **22**: 143-50.

21. Margolis DJ, Allen-Taylor L, Hoffstad O, Berlin JA. The accuracy of venous leg ulcer prognostic models in a wound care system. *Wound Repair Regen* 2004; **12**: 163-8.

22. Moore K, Huddleston E, Stacey MC, Harding KG. Venous leg ulcers - the search for a prognostic indicator. *Int Wound J* 2007; **4**: 163-72.

23. van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M*, et al.* Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 2002; **415**: 530-6.

24. Sparano JA, Gray RJ, Makower DF, Pritchard KI, Albain KS, Hayes DF*, et al.* Prospective Validation of a 21-Gene Expression Assay in Breast Cancer. *N Engl J Med* 2015; **373**: 2005-14.

25. Panuncialman J, Hammerman S, Carson P, Falanga V. Wound edge biopsy sites in chronic wounds heal rapidly and do not result in delayed overall healing of the wounds. *Wound Repair Regen* 2010; **18**: 21-5.

26. Senet P, Combemale P, Debure C, Baudot N, Machet L, Aout M*, et al.* Malignancy and chronic leg ulcers: the value of systematic wound biopsies: a prospective, multicenter, cross-sectional study. *Arch Dermatol* 2012; **148**: 704-8.

27. Pedersen TX, Leethanakul C, Patel V, Mitola D, Lund LR, Dano K*, et al.* Laser capture microdissection-based in vivo genomic profiling of wound keratinocytes identifies similarities and differences to squamous cell carcinoma. *Oncogene* 2003; **22**: 3964-76.

28. Stojadinovic O, Brem H, Vouthounis C, Lee B, Fallon J, Stallcup M*, et al.* Molecular pathogenesis of chronic wounds: the role of beta-catenin and c-myc in the inhibition of epithelialization and wound healing. *Am J Pathol* 2005; **167**: 59-69.

29. Nassiri S, Zakeri I, Weingarten MS, Spiller KL. Relative Expression of Proinflammatory and Antiinflammatory Genes Reveals Differences between Healing and Nonhealing Human Chronic Diabetic Foot Ulcers. *J Invest Dermatol* 2015; **135**: 1700-03.

30. Serena TE, Cullen BM, Bayliff SW, Gibson MC, Carter MJ, Chen L*, et al.* Defining a new diagnostic assessment parameter for wound care: Elevated protease activity, an indicator of nonhealing, for targeted protease-modulating treatment. *Wound Repair Regen* 2016; **24**: 589-95.

31. Cole J, Tsou R, Wallace K, Gibran N, Isik F. Early gene expression profile of human skin to injury using high-density cDNA microarrays. *Wound Repair and Regeneration* 2001; **9**: 360-70.

32. Bernard FX, Pedretti N, Rosdy M, Deguercy A. Comparison of gene expression profiles in human keratinocyte mono-layer cultures, reconstituted epidermis and normal human skin; transcriptional effects of retinoid treatments in reconstituted human epidermis. *Exp Dermatol* 2002; **11**: 59-74.

33. Tomic-Canic M, Brem H. Gene array technology and pathogenesis of chronic wounds. *Am J Surg* 2004; **188**: 67s-72s.

34. Stojadinovic O, Brem H, Vouthounis C, Lee B, Fallon J, Stallcup M*, et al.* Molecular pathogenesis of chronic wounds: the role of beta-catenin and c-myc in the inhibition of epithelialization and wound healing. *Am J Pathol* 2005; **167**: 59-69.

35. Wu SC, Marston W, Armstrong DG. Wound care: the role of advanced wound healing technologies. *J Vasc Surg* 2010; **52**: 59-66.

36. Brem H, Stojadinovic O, Diegelmann RF, Entero H, Lee B, Pastar I*, et al.* Molecular markers in patients with chronic wounds to guide surgical debridement. *Mol Med* 2007; **13**: 30-9.

37. Gohel MS, Heatley F, Liu X, Bradbury A, Bulbulia R, Cullum N*, et al.* A Randomized Trial of Early Endovenous Ablation in Venous Ulceration. *N Engl J Med* 2018; **378**: 2105-14.

38. Coerper S, Beckert S, Kuper MA, Jekov M, Konigsrainer A. Fifty percent area reduction after 4 weeks of treatment is a reliable indicator for healing--analysis of a single-center cohort of 704 diabetic patients. *J Diabetes Complications* 2009; **23**: 49-53.

FIGURES

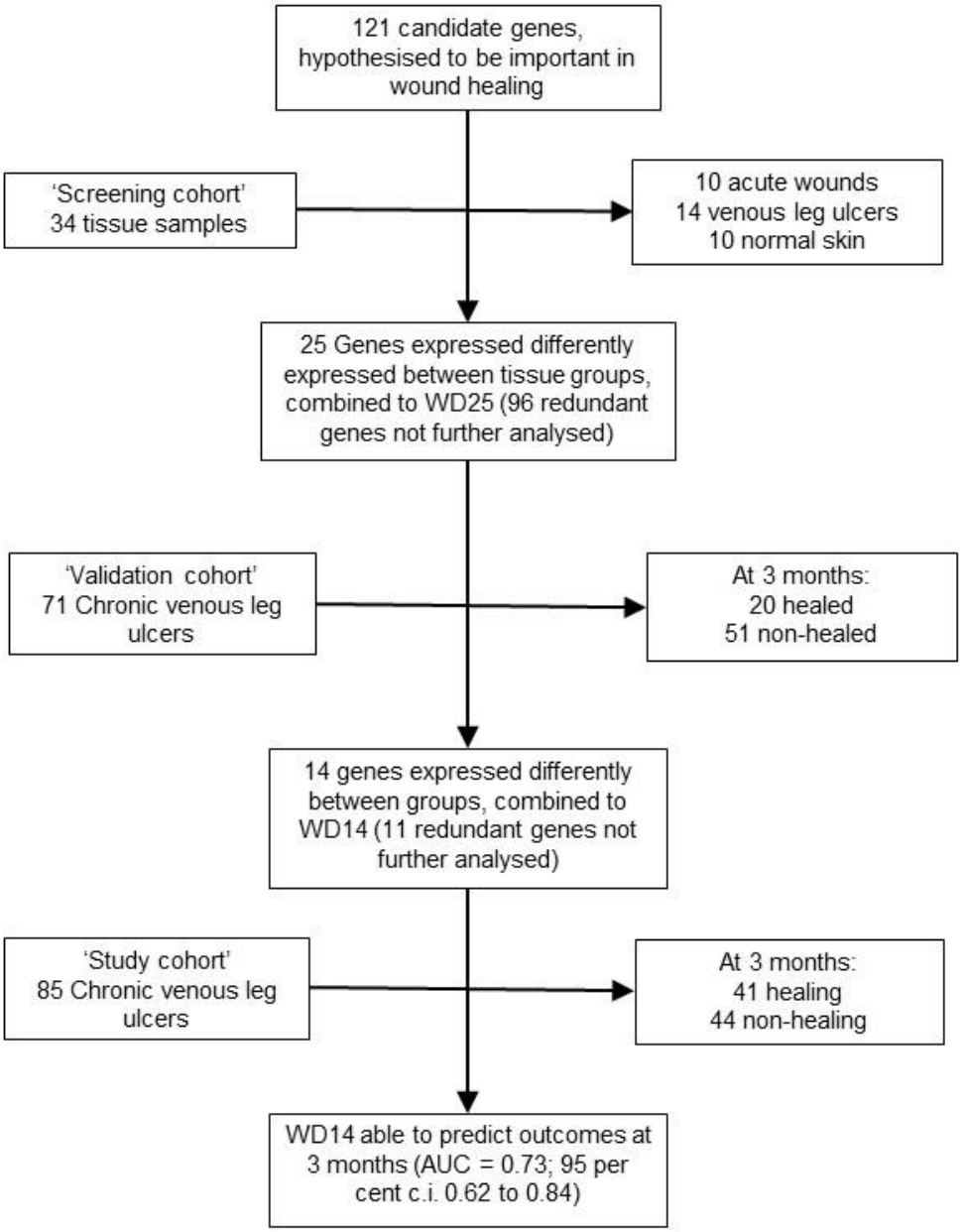


Figure 1

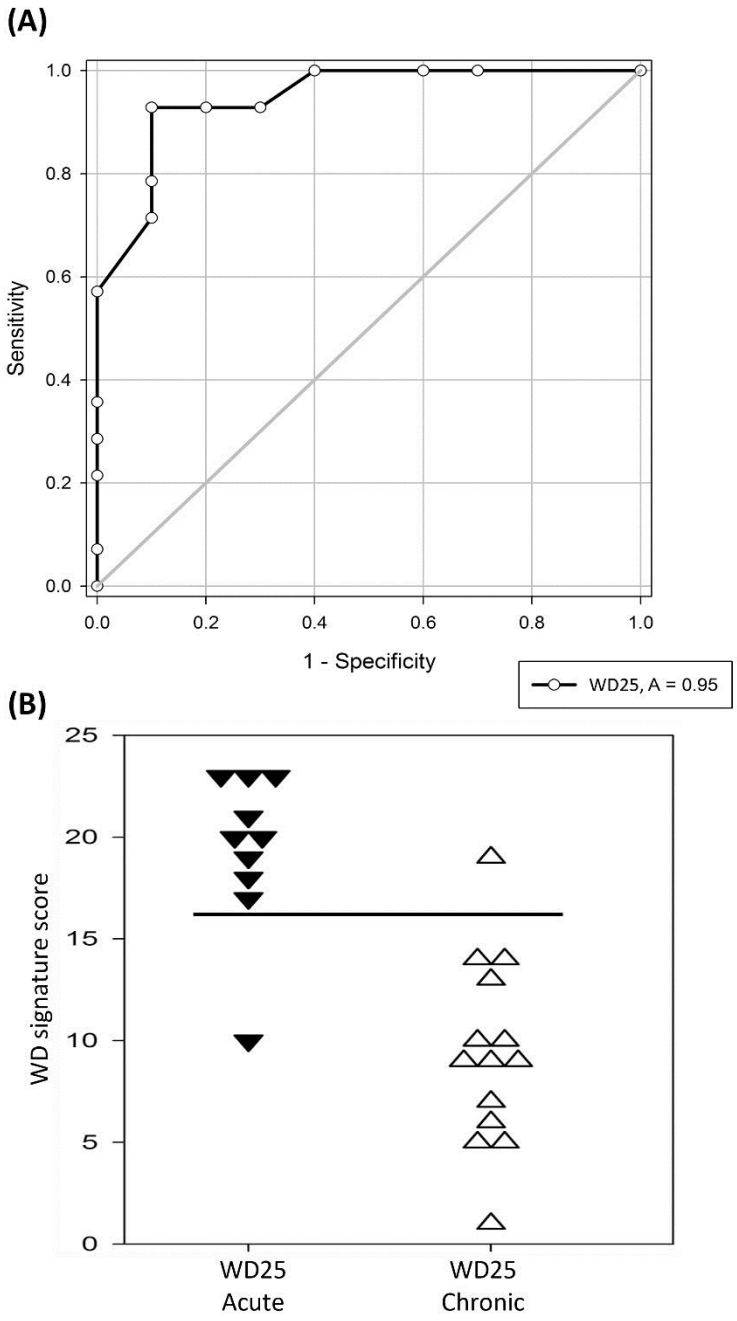

(A)
Sensitivity
1 - Specificity
0.0
0.2
0.4
0.6
0.8
1.0
WD25, A = 0.95
(B)
WD signature score
0
5
10
15
20
25
WD25
Acute
WD25
Chronic

Figure 2

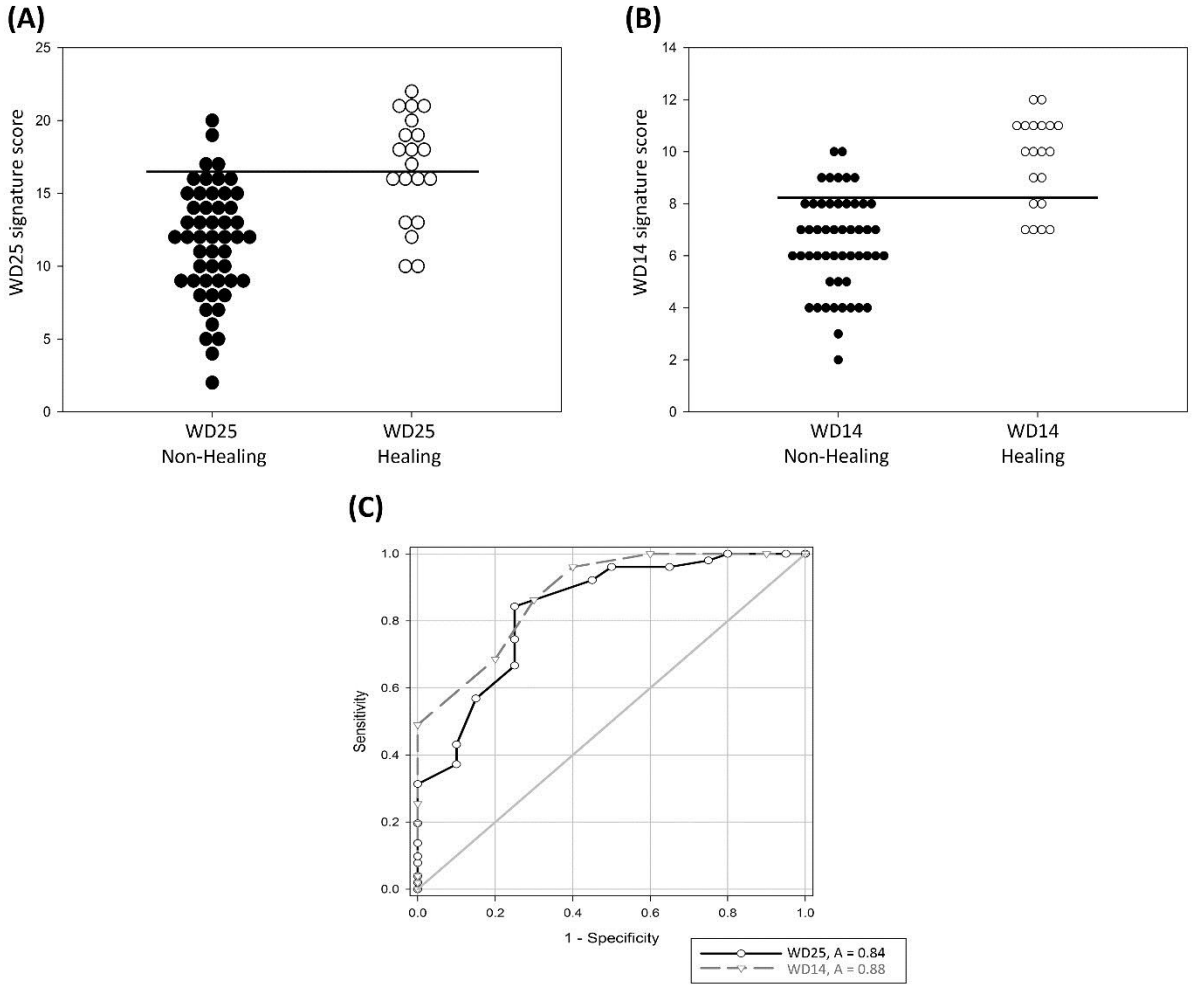
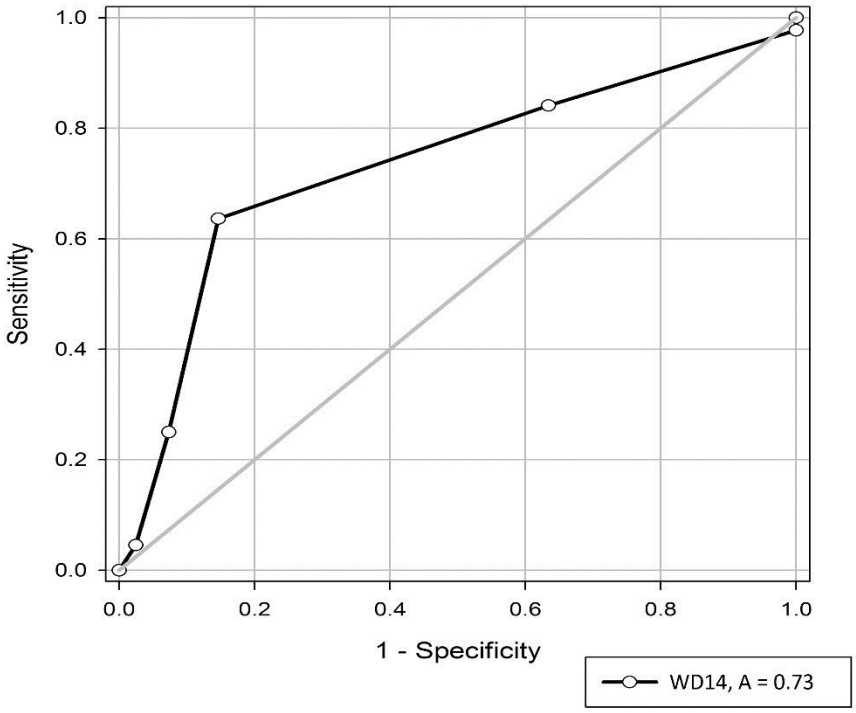

(A)
WD25 signature score
25
20
15
10
5
0
WD25
Non-Healing
WD25
Healing
(B)
WD14 signature score
14
12
10
8
6
4
2
0
WD14
Non-Healing
WD14
Healing
(C)
Sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
1 - Specificity
WD25, A = 0.84
WD14, A = 0.88

Figure 3

(A)


1.0
0.8
0.6
0.4
0.2
0.0
Sensitivity
0.0
0.2
0.4
0.6
0.8
1.0
1 - Specificity
WD14, A = 0.73

(B)

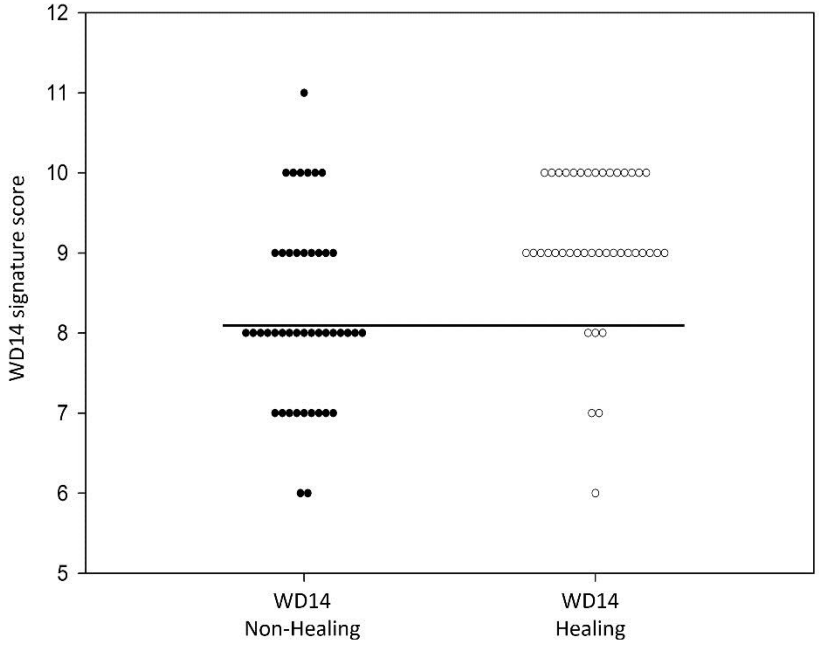

12
11
10
9
8
7
6
5
WD14 signature score
WD14
Non-Healing
WD14
Healing

Figure 4

FIGURE LEGENDS

Figure 1: Flow diagram outlining study design and development of WD14 signature.

Figure 2: ROC analysis of WD25 signature in a clinical cohort of acute and chronic wounds. (A) ROC curve and (B) boxplot presentation of WD25 within the acute (n=10) and chronic (n=14) wound cohort. Signature score cut off value (>16) indicated on box plot.

Figure 3: ROC analysis and comparison of WD25 and WD14 predictive power in chronic healing and non-healing wound cohort after 12 weeks. Boxplot presentation of (A) WD25 signature scores and (B) WD14 signature scores within the chronic healing (n=20) and non-healing (n=51) clinical wound cohort. Signature score cut off values (A; >16 and B; >8) indicated on box plots. (C) ROC curve and AUC comparison of the WD25 and WD14 predictive tests demonstrating a slight improvement with the WD14 gene expression signature.

Figure 4: ROC analysis of WD14 signature within the study cohort. (A) ROC curve and (B) boxplot presentation of WD14 within the healing (n=41) and non-healing (n=44) study cohort. Signature score cut off value (>8) indicated on box plot.

Development and validation of a gene expression test to identify hard-to-heal chronic venous leg ulcers; a three-stage cohort study

SUPPLEMENTARY MATERIAL

Supplementary information

Processing of biopsies for RNA extraction

Wound biopsies were sectioned on a Leica cryostat (Leica Microsystems Ltd,. Milton Keynes, UK) to a 7µm thickness. For RNA extraction, 50-75 sections were combined and homogenised in an RNA extraction solution (TRI Reagent®, Sigma-Aldrich, UK). cDNA was generated from the same amount of RNA using a reverse transcription kit (Primer Designs, Southampton, England). For the screening and validation cohort, cDNA samples were randomly plated in a 96-well plate (Advanced Biotechologies Ltd. (Abgene), Epsom, Surrey, UK) for high throughput screening, whilst samples from the study cohort were individually stored for batch testing with the WD14 gene signature.

Quantitative gene transcript analysis

Quantitative polymerase chain reaction (QPCR) gene transcript analysis was undertaken using the Amplifluor^{TM} Universal Detection System (Intergen Inc, Oxford, UK). Specific primer sets were designed to amplify a unique region of each target gene transcript and tagged with an additional Z-sequence at the 5'-end of one of the primers, which links to the complementary FAM-tagged probe, Uniprimer^{TM} (Intergen Inc, Oxford, UK). Primers were synthesised by Sigma Genesis (Poole, UK, Table S6. In a typical reaction, QPCR mastermix (IQ supermix; BioRad Laboratories, Hemel Hempstead, UK), the primer pair, probe and cDNA from samples (or negative/positive control template) were included and the reactions run on an iCycler real time PCR detection system (BioRad Laboratories, Hemel Hempstead, UK). cDNA was denatured and the polymerase activated for 12 minutes at 95^{o}C, followed by

100 cycles of: denaturing (95^{o}C for 15 seconds), annealing (55^{o}C for 20 seconds) and synthesis (72^{o}C for 15 seconds). The detection of fluorescent signal was carried out at the annealing stage and run simultaneously with an internal standard, a purified plasmid coding podoplanin, allowing for the calculation of relative expression within the samples in each test plate. Such technology has previously been utilised by our group and reported[1,2]. Three housekeeping genes, cytokeratin-19, β-actin and GAPDH, were used in all assays.

Table S1 Demographic data for patients in the screening cohort.

Wound type	Age (Median; range)	Sex (per cent male)
Acute wound	22 (19.5-25.5.)	70
Chronic wound	69 (64-73)	58.8
Normal skin	24.5 (22.3-31.8)	30

Table S2. List names, and accession numbers of gene transcripts tested.

The biological similarities of cancer and wound healing have been well documented[3,4]. It was hypothesised that genes promoting cancer progression would also be enhanced in wounds undergoing healing.

Gene	**Accession number**
AAMP	M95627
Actin	NM_001101
AMFR	L35233
ARP2	AF006082
BMP10	NM_014482
BMP15	NM_005448
BMP7	BC004248
BMP8	NM_181809
BMP9	AF188285
CAR1	NM_001338
CCN2	NM_001901
CCN3	NM_002514
CD24	BC064619
CD34	M81104 X60172
CD49F	NM_002203
Chordin V1	AF209929
Chordin V3	AF283325
Chordinv2	AF209930
Claudin-5	NM_003277

CMG1	AY040325
CMG2	AY040326
COM1	NM_012385
CREBL1	NM_004381
Cyr61	AF307860
DRIM	NM_014503
EHM2	AB032179
Endomucin-2	AB034695
FAP	U09278
GAPDH	NM_002046
GDF9A	NM_005260
HGFL	NM_020998
IL13	U70981
IL17A	NM_002190
IL17B	NM_014443
IL17BR	AF212365
IL17C	NM_013278
IL22R	BC029273
IL24	BC009681
IL4	M13982

IL8R	U58828
IL8RB	NM_001557
JAK1	M64174 M35203
KAI1	U20770
KISS1	AY117143
Kiss1R	NM_032551
L1CAM	M77640
LYN	BC068551
NOTICH1	AF308602
N-WASP	D88460
OSPA	NM_001040058
OSP-C	NM_001040060
PAR4	AB108448
PEDF	M76979
PlGF1	X54936
Psoriasin	M86757
PTPRK	AF533875
RGMa	NM_020211
RGMc	BC085604
RHO GDI-G	AF498928
RHO-8	AF498969
RHO-C	L25081
ROCK1	D87931
RON	NM_002447

SATB1	NM_002971
SATB2	NM_015265
SDF1	XM_165565
SHH	L38518
SNAIL	AF131208
ß-Catenin	P35222
SSTR1	L14865
STYK1	NM_018423
TEM1	XM_006495
TEM4	AF378754
TEM6	AF378756
TEM7R	AF378757
TEM8	NM_032208
VEGF	E14233
VEGF-C	AF244813
VEGF-D	D89630
VEGF-R	E13256
VEGF-R2	AF063658
WAVE1	AF134303
WAVE2	AB026542
WAVE3	AB026543
HGF	X16323
HGFA	D14012
cMET	J02958

VEGI	BD131562
ADAM1	NR_036636
ADAM10	NM_001110
ATF1	NM_005171
BDNF	AF400438
Neuropilin-1	AF018956
Neuropilin-2	AF022859
FAM3C	BC046932
TANK	U63830
Aurora-A	D84212
Aurora-B	NM_004217
PPAR-gamma	NM_015869
P53	M14695
LOX	M94054
LOX-L2	NM_002318
LOX-L4	AF395336
EPLIN	AF198454

HuR	U38175
STYK1	NM_018423
DR3	U72763
DcR3	AF104419
ARP2	AF006082
IL22	AF279437
Matrip2	AJ319876
Matrip1	AF118224
HAI1	NM_003710
HAI2	E12900
SOCS-1	NM_003745
SOCS-2	BC010399
SOCS-3	NM_003955
SOCS-4	BC060790
SOCS-5	NM_144949
SOCS-6	NM_004232
SOCS-7	XM_371052

Table S3. The 25 gene signature list, comprising genes whose expression was significantly altered within a cohort of acute and chronic wounds and normal skin. Median, together with Q1 and Q3 values are shown. Based on their differential expression, together with our prior knowledge, genes were designated either 'promoting' of 'inhibiting' of wound healing.

Molecule name	**Median (Q1-Q3) expression in Acute wounds (n = 10)**	**Median (Q1-Q3) expression in Chronic wounds (n =14)(Q1-Q3)**	**Median (Q1-Q3) expression in normal skin (n = 10)**	**p-value (Kruskal Wallis)**	**Expressional differences between wound and normal tissues**	**Predicted effect on the wound healing**
AMFR	1201 (825-8139)	281 (197-578)	1097 (257-2339)	0.024	Enhanced expression in acute wounds compared to chronic	Promoting
ARP2	74 (32-236)	2.5 (0.75-5.50)	5 (0-28.5)	0.000	Decreased in chronic and increased in acute wounds compared to normal skin	Promoting
β-Catenin	761 (1-29948)	0 (0-1)	1 (0-58379)	0.026	Decreased in chronic wounds compared to acute wounds	Promoting
BMP15	1 (0-3)	0 (0-0)	0 (0-1)	0.068	Decreased in chronic wounds compared to acute wounds	Promoting
CAR1	1676 (255-9537)	128 (9.2-226.7)	35 (0-292)	0.001	Decreased in chronic and normal skin compared to acute wounds	Promoting
Claudin-5	14 (5-86)	0 (0-0.25)	0 (0-1.5)	0.001	Increased in acute wounds compared to normal skin and chronic wounds	Promoting
CREB1l	142 (53-454)	15.5 (8.25-38.25)	35 (6-227)	0.004	Decreased in chronic and increased in acute wounds compared to normal skin	Promoting
Endomucin-2	1197 (410-3357)	468 (210–3805)	63713 (7835-221687)	0.006	Decreased in chronic wounds	Promoting

					compared to acute wounds	
IL17BR	0.055 (0.023-1.18)	0.0028 (0.0006-0.01)	0.005 (0.002-0.043)	0.015	Decreased in chronic and increased in acute wounds compared to normal skin	Promoting
IL17C	0 (0-1)	0.01 (0.0075-0.0350)	0.025 (0-0.138)	0.027	Decreased in chronic and acute compared to normal skin	Promoting
IL22R	1555 (505-3434)	302 (143-627)	0 (0-175.5)	0.000	Increased in both acute and chronic wounds compared to normal skin compared to normal skin	Promoting
IL8RB	34 (3-85)	0.5 (0-1.125)	0.5 (0-11)	0.001	Decreased in chronic and normal skin compared to acute wounds	Promoting
KAI1	395 (161-11885)	71.5 (37-105)	0 (0-141)	0.001	Increased in both acute and chronic wounds compared to normal skin	Inhibiting
N-WASP	0.115 (0.016-0.332)	0.019 (0.0012-0.06)	0.02 (0.005-0.049)	0.059	Decreased in chronic and normal skin compared to acute wounds	Promoting
Par4	7 (2-47)	0 (0-2)	2 (0-5)	0.011	High in acute wounds compared to chronic wounds	Promoting
PEDF	411 (22-1858)	11 (4-125)	1204 (47-4580)	0.011	Decreased in human wounds compared to normal skin	Promoting
Psoriasin	23891 (326-337629)	2 (0-157)	1 (0-13045)	0.01	Decreased in chronic and normal skin compared to acute wounds	Promoting
PTPRK	132 (37-464)	2 (1-9)	0 (0-50)	0.001	Increased in acute and chronic wounds compared to normal skin	Promoting
RhoGDI-G	31 (14-478)	2 (0.8-12.5)	166 (13-1145)	0.009	Decreased in human wounds	Inhibiting

					compared to normal skin	
RON	1 (0-2)	0 (0-0.1)	0 (0-0.45)	0.028	Increased in acute wounds compared to chronic and normal skin	Promoting
TEM4	135 (63-394)	14 (6-33.75)	40 (14.3-136)	0.001	High in acute wounds compared to chronic wounds	Promoting
TEM7R	11 (8-64)	3.5 (1-4.5)	0 (0-5.5)	0.001	Increased in both acute and chronic wounds compared to normal skin	Promoting
VEGF-C	16 (9-92)	2.5 (0.75-6.25)	7 (3.5-72.7)	0.007	Decreased in chronic wounds compared to acute wounds	Promoting
VEGF-D	2 (0-19)	0 (0-0.25)	0 (0-2)	0.021	Decreased in chronic and normal skin compared to acute wounds	Promoting
WAVE2	0.020 (0.01-0.59)	0.009 (0.0004-0.012)	0.017 (0.0017-0.06)	0.275	High in acute wounds compared to chronic wounds	Promoting

Table S4. Composition of the WD14 gene signature. Genetic markers were divided into two groups depending on their anticipated healing impact.

	Gene
Promoting wound healing	ARP2
	CAR1
	Claudin-5
	CREBL1
	Endomucin-2
	IL8RB
	IL17BR
	IL22R
	Psoriasin
	PTPRK
	TEM4
	TEM7R
	VEGF-C
Inhibiting wound healing	KAI1

Table S5 Demographics of cohort used for WD14

	Median	Inter-quartile range
Age	75.0 (years)	63 to 83
Wound duration	21.0 (months)	9.0 to 49.5
	Frequency	
Sex (male)	40.2 per cent	
Smokers (active)	22.2 per cent	
Rheumatoid Arthritis	9.7 per cent	
Connective Tissue Disease	1.4 per cent	
Immunosupression	11.3 per cent	
Active cancer	7.0 per cent	
Malnutrition	10.6 per cent	

Table S6 Primers for the genes comprising the 25 gene signature list (WD25). Primer pairs used to detect the GAPDH, CK-19 and Actin housekeeping genes are also listed.

Primer set	**Forward**	**Reverse**
IL8rb	CGTTACCTGGCCATTGTC	*ACTGAACCTGACCGTACA*GCAGGGACAGATTCATAGAC
PEDF	GGTGCTACTCCTCTGCATT	*ACTGAACCTGACCGTACA*AGAAAGGATCCTCCTCCTC
CL5	TTCCTGGACCACAACATC	*ACTGAACCTGACCGTACA*CACCGAGTCGTACACTTTGC
RON	CATCCACCCAGTGCCAAC	*ACTGAACCTGACCGTACA*CCACACAGTCAGCCACAG
KAI1	CATTCGAGACTACAACAGCA	*ACTGAACCTGACCGTACA*TCCAGTTGTAGAAGCTGACC
RhoGDI gamma	AGTCCTCCTGGCTGACAA	*ACTGAACCTGACCGTACA*CACAGCCTCATCCAACAC
N-WASP	AGTCCCTCTTCACTTTCCTC	*ACTGAACCTGACCGTACA*AGATCTCTGTGGATTGTCCT
Endomucin-2	AAATGTTGTCACACCAACAA	*ACTGAACCTGACCGTACA*AGCTGTTGACATCAGAGACA
PTPRK	TATGGCTGTACCTCCATTGT	*ACTGAACCTGACCGTACA*ATATCGTAGCATCCCTTCCT

β-CATENIN	AGGGATTTTCTCAGTCCTTC	*ACTGAACCTGACCGTACA*CATGCCCTCATCTAATGTCT
IL17C	CATCTCACCCTGGAGATACC	*ACTGAACCTGACCGTACA*CATCGATACAGCCTCTGC
VEGFD	GCTCCAGTAATGAACATGG	*ACTGAACCTGACCGTACA*ATCTGCTGTTCAGATCGTT
WAVE 2	CAGCTGACTACCCAACTGTG	*ACTGAACCTGACCGTACA*ATCTGCACCAGTGAAAGG
TEM4	GTCTCGTTCAAGCTGCTG	*ACTGAACCTGACCGTACA*GGTGTCCGTGTCCTCCTC
BMP 15	GTGAACCCCTTGACCAGT	*ACTGAACCTGACCGTACA*TTGGTATAGTCCTCGGTTTG
Psoriasin (S100A7)	AACTTCCCCAACTTCCTTAG	*ACTGAACCTGACCGTACA*AGCAAGGACAGAAACTCAGA
IL17BR	AGTGACTGGGGATAGTGAAG	*ACTGAACCTGACCGTACA*CAGAGCACAACTGTTCCTTT
TEM7R	CTTGATTGGCAGTATGGAGT	*ACTGAACCTGACCGTACA*GTCTACCGCCTTGAGAAAG
CAR1	ATGGATCTGAAGAAATTGGA	*ACTGAACCTGACCGTACA*AGACAATTTTTGCCACTCAT

AMFR	GAAGGTGCGTCCTCTGAC	*ACTGAACCTGACCGTACA*TAGGAGGTCTGCTGCTTCT
IL22R	AGATGACTGACAGGTTCAGC	*ACTGAACCTGACCGTACA*GAATCGATCTCACTTTGGAG
CREBL1	GGGGACTATGAGGAGATGAT	*ACTGAACCTGACCGTACA*GTGGAGGTCTTGATGTGAAT
PAR4	*ACTGAACCTGACCGTACA*GATCTTACGCTTCCCTTACC	ATGCCAGGAGACGACCTC
VEGFC	GCTGCTGCACATTATAACAC	*ACTGAACCTGACCGTACA*AACTCCTTCCCCACATCTAT
ARP2	ATTGAGCAAGAGCAGAAACT	*ACTGAACCTGACCGTACA*TTCTGGTGCTTCAAATCTCT
GAPDH	CTGAGTACGTCGTGGAGTC	*ACTGAACCTGACCGTACA*CAGAGATGATGACCCTTTTG
CK19	CAGGTCCGAGGTTACTGAC	*ACTGAACCTGACCGTACA*CAGTTTCTGCCAGTGTGTCTTC
ACTIN	CATTAAGGAGAAGCTGTGCT	*ACTGAACCTGACCGTACA*GCTCGTAGCTCTTCTCCAG

References

1. Harries RL, Owen S, Ruge F, Morgan M, Li J, Zhang Z*, et al.* Impact of pigment epithelium-derived factor on colorectal cancer in vitro and in vivo. *Oncotarget* 2018; **9**: 19192-202.
2. Li J, Ye L, Sun PH, Zheng F, Ruge F, Satherley LK*, et al.* Reduced NOV expression correlates with disease progression in colorectal cancer and is associated with survival, invasion and chemoresistance of cancer cells. *Oncotarget* 2017; **8**: 26231-44.
3. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med* 1986; **315**: 1650-9.
4. Schafer M, Werner S. Cancer as an overhealing wound: an old hypothesis revisited. *Nat Rev Mol Cell Bio* 2008; **9**: 628-38.